CD81 and CD28 Costimulate T Cells Through Distinct Pathways[1]

Deborah A. Witherden, Richard Boismenu, and Wendy L. Havran[2]

We have examined the role of CD81 in the activation of murine splenic $\alpha\beta$ T cells. Expression of the CD81 molecule on T cells increases following activation, raising the possibility of a role for this molecule in progression of the activation process. Using an in vitro costimulation assay, we show that CD81 can function as a costimulatory molecule on both $CD4^+$ and $CD8^+$ T cells. This costimulation functions independently of CD28, and unlike costimulation through CD28, is susceptible to inhibition by cyclosporin A. Strikingly, the pattern of cytokine production elicited by costimulation via CD81 is unique. IL-2 production was not up-regulated, whereas both IFN-γ and TNF-α expression significantly increased. Together our results demonstrate an alternate pathway for costimulation of T cell activation mediated by CD81. *The Journal of Immunology,* **2000, 165: 1902–1909.**

T cell activation requires the coordinate interaction between molecules on T cells and those on APCs. At least two independent signals are critical for the generation of an effective T cell response. The first is generated by interaction between the TCR on the T cell and MHC plus peptide on the APC. The second signal is also generated by interaction between molecules on T cells and those on APCs, and provides the necessary costimulatory signal to generate a complete T cell immune response (1–3). The best characterized of the molecules eliciting this costimulatory signal are the B7 family members on APCs and CD28 on T cells (4–6). The interaction between CD28 and B7-1 (CD80) or B7-2 (CD86) in conjunction with TCR engagement has been shown to elicit Ag-specific T cell responses as well as allogenic mixed lymphocyte reactions. This response can also be mimicked in vitro using Abs specific for CD3 and CD28.

Ligation of the TCR initially results in full phosphorylation of tyrosine residues in the ζ-chain of the CD3 complex, leading to recruitment and activation of nonreceptor tyrosine kinases such as ZAP-70 (7). Here, intracellular signaling pathways diverge, but they ultimately converge once again in the nucleus. Evidence indicates that the signal transduction pathway regulated by CD28 is distinct from that generated through the TCR (5, 8). Although not yet fully understood, signals through CD28 appear to be mediated by phosphatidylinositol (PI)[3] 3-kinase (5, 9–13) and sphingomyelinase (14, 15), and also involve many other signaling cascades including activation of phospholipase Cγ (16–18), $p21^{ras}$ (19), and c-Raf-1 (20). CD28 pathways are believed to not control gene transcription directly, but to converge with TCR signals to fully activate transcription factors and thereby induce cytokine synthesis. One model suggests that both pathways are integrated on the level of the protein kinases JNK1 (c-Jun N-terminal kinase) and JNK2, because these kinases need both TCR and CD28 signals for full activation (21). A recent report suggests that CD28 in fact amplifies TCR-induced ZAP-70 activity, thereby regulating the intersection of the TCR and CD28 signaling pathways (22). Another school of thought favors CD28 engagement leading to the redistribution and clustering of membrane and intracellular kinase-rich raft microdomains at the site of TCR engagements, resulting in higher and more stable tyrosine phosphorylation of substrates (23).

Despite the tremendous focus on CD28, analysis of mice deficient for this molecule indicated that CD28 was in fact not the only molecule capable of providing a costimulatory signal to T cells. Although CD28-deficient mice do show a reduced response to lectins and T helper activity, they can mount efficiently an immune response to virus and are able to reject allografts (24, 25). Clearly, other molecules can provide costimulatory activity for T cells. Several molecules have in fact been identified, including CD43 (26), HSA (27), CD2 (28, 29), CD5 (30, 31), CD44 (32), CD29 (33, 34), CD11a (28, 35), members of the TNF receptor family such as 4-1BB (36), GPI-anchored molecules such as Thy-1 (37), and the tetraspanins CD82 (38) and CD9 (39, 40). More recently, two novel molecules have been described, the signaling lymphocytic activation molecule (SLAM) and inducible costimulator (ICOS), both of which appear to play a role in T cell activation distinct from that of CD28 (41–43). The precise mechanism of costimulation by any of these molecules is unknown and at present, for the majority of these molecules, it is unknown whether or not they function independently of CD28.

CD81, also known as TAPA-1 (target of antiproliferative Ab-1), is a member of the tetraspanin or transmembrane 4 superfamily (TM4SF) (44, 45). It is expressed on a wide variety of tissues and cell types, including both B and T cells as well as epithelial cells, and has the capacity to associate with other cell surface proteins in a cell type-specific manner (46, 47). On B cells, CD81 forms part of the CD19/CD21 B cell Ag receptor-coreceptor complex (48–50). This complex functions in a similar manner to that of CD4 or CD8 in T cells, lowering the threshold for activation (51). In fact, mice deficient for the CD81 molecule show decreased expression of this CD19/CD21 complex and subsequently reduced B cell proliferation and Ab production (52–54).

In human T cell lines, CD81 can associate with the CD4 and CD8 coreceptors (55, 56). Interestingly, CD81 appears to bind to

Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037

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[2] Address correspondence and reprint requests to Dr. Wendy L. Havran, Department of Immunology, IMM8, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037. E-mail address: havran@scripps.edu

[3] Abbreviations used in this paper: PI, phosphatidylinositol; TM4SF, transmembrane 4 superfamily; CsA, cyclosporin A; RPA, RNase protection assay; PTK, protein tyrosine kinase; TSRI, The Scripps Research Institute.

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the cytoplasmic region of CD4, and its binding is inhibited by bound $p56^{lck}$ (56). However, the function of this association with CD4 and CD8 is unknown. What is known is that CD81 expression increases after mitogenic T cell activation (W. L. Havran and R. Boismenu, unpublished observations). Furthermore, CD81 expressed on human thymocytes can function as a costimulatory molecule for these cells, with simultaneous cross-linking of CD81 and CD3 promoting a vigorous proliferative response (57).

In fact, while structurally distinct from CD28, several features of CD81 do suggest an important intracellular signaling function for this molecule. It has hydrophilic amino- and carboxyl-terminal cytoplasmic domains and four membrane-spanning regions (46, 58). Most sequence diversity found between species is contained within the large extracellular loop located between the third and fourth transmembrane regions (47). The transmembrane domains and cytoplasmic regions are highly conserved (47). CD81 lacks immunoreceptor tyrosine-phosphorylated activation motifs, thus the possible mechanism of signaling by the CD81 molecule remains to be defined.

CD81 is also associated with a number of integrins, including $\alpha_3\beta_1$ (59) and $\alpha_6\beta_1$ (60), and recent evidence suggests that TM4SF proteins may in fact act as linkers between the extracellular α-chain domains and intracellular signaling molecules such as PI 4-kinase and protein kinase C (61–63). It is thus possible that CD81 and other tetraspanins do not in fact signal, but exert, their effects by inducing signaling through their associated molecules.

In this report, we demonstrate that murine CD81 can indeed function as a T cell costimulatory molecule on both the $CD4^+$ and $CD8^+$ subsets of $\alpha\beta$ T cells. This costimulation functions independently of CD28, and in fact appears to follow a different pathway to T cell activation resulting in the generation of a cytokine profile distinct from that elicited by CD28-mediated costimulation. This suggests that CD81 may play a unique role in $\alpha\beta$ T cell immune responses.

Materials and Methods

Mice

C57BL/6 mice were raised in our breeding colony at The Scripps Research Institute (TSRI; La Jolla, CA). Mutant mice with a disrupted CD28 gene ($CD28^{0/0}$; Ref. 24) were obtained from The Jackson Laboratory (Bar Harbor, ME) and had been backcrossed several generations onto the C57BL/6 background. $RAG^{0/0}$DO11.10 TCR transgenic mice (64) obtained from Dr. S. Webb (TSRI) and $RAG^{0/0}$OT-1 TCR transgenic mice (65) obtained from Dr. C. Surh (TSRI) were used as a source of exclusively naive $CD4^+$ and $CD8^+$ T cells, respectively. In all experiments, mice were used at 6–8 wk of age.

Antibodies

Anti-CD3ϵ mAb (2C11), anti-CD4, and anti-CD8 were obtained from PharMingen (San Diego, CA) and used as purified Ab. The anti-CD28 (37.51) was kindly provided by Dr. James Allison and has been described previously (66). 2F7 is a hamster mAb produced in our laboratory, directed against murine CD81 (67). 1F3 is a hamster mAb produced from the same fusion as the 2F7 mAb. It recognizes an unknown Ag on epithelial cells, not expressed on lymphocytes. Either 1F3 or hamster Ig were used as the control Ab in all experiments. Purified anti-mouse IgG + IgM Abs used for B cell depletions were obtained from Caltag (Burlingame, CA). Anti-CD4, -CD8, -CD25, -CD69, and -Thy1.2 Abs used for FACS analysis were obtained from PharMingen directly conjugated to FITC, PE, Cychrome, or allophycocyanin.

T cell purification

Single cell suspensions of spleen cells were isolated on density gradients of Lympholyte-M (Cedarlane Laboratories, Ontario, Canada). T cells were purified from these suspensions by depletion of B cells on anti-mouse IgG- + IgM-coated flasks as described previously (68). Briefly, spleen cell suspensions were incubated at room temperature for 1 h in flasks that had been coated overnight with anti-mouse IgG + IgM Abs (Caltag). The purity of the cell population harvested from the flasks was analyzed by flow cytometry. Typically >95% of recovered cells were $Thy1^+$ and >90% $CD44^{low}$.

For the purification of $CD4^+$ and $CD8^+$ subsets of T cells following panning, T cells were further purified using negative selection on magnetic beads. Briefly, T cells were incubated with purified anti-mouse CD4 mAb (RM4-5; PharMingen) or anti-mouse CD8 mAb (53.6.7; PharMingen) for 30 min on ice. Cells were washed once with DMEM supplemented with 2% FCS and then incubated with anti-rat Ig-coated magnetic beads (BioMag; PerSeptive Biosystems, Framingham, MA) at 1 ml beads per 10^7 cells for 30 min on ice with occasional mixing. Magnetic bead-coated cells were then removed using a magnet (Advanced Magnetics; Cambridge, MA), and the unbound cells were washed once in DMEM supplemented with 2% FCS. Purity of the population was assessed by flow cytometry. Typically, purified $CD4^+$ cells contained <1% $CD8^+$ cells and purified $CD8^+$ cells contained <3% $CD4^+$ cells.

Costimulation assays

Abs were diluted in ELISA coating buffer (50 mM Tris, 150 mM NaCl; pH 8.0 at room temperature) and immobilized to individual wells of 96-well flat-bottom microtiter ELISA plates in a final volume of 100 μl. The plates were incubated at 4°C overnight. Before the addition of cells, the plates were washed twice with ELISA coating buffer and blocked for 15 min with 100 μl DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 10 mM HEPES, and 50 μM 2-ME.

Purified T cells or T cell subsets were cultured at 1×10^5 cells per well. In some cases cyclosporin A (CsA) (Sigma, St. Louis, MO) or herbimycin A (Sigma) were also added at various concentrations. Unless otherwise stated, cells were pulsed with 0.5 μCi [^{3}H]thymidine at 58 h after initiation of culture, and harvested 14 h later. For time course experiments, [^{3}H]thymidine was added for the final 14 h of each time point. Cells were harvested onto glass fiber filters (Cambridge Technology, Watertown, MA) and 2 ml scintillation fluid was added to each sample. Counts were read on a Beckman LS3801 scintillation counter (Beckman Coulter, Fullerton, CA). Cell viabilities were determined at various time points by trypan blue dye exclusion. All data points were performed in triplicate and are presented as mean ± SD.

Immunofluorescence staining and flow cytometry

Cells were harvested from in vitro cultures 48 h following the initiation of culture. Cells were washed once in PBS containing 2% FCS and 0.02% sodium azide (wash buffer), and resuspended in wash buffer. The following mAbs were used for immunofluorescence staining as FITC, PE, or Cychrome conjugates: CD4, CD8, CD25, and CD69. mAbs to CD28 and CD81 were used conjugated to biotin and revealed with streptavidin-coupled Red670. Cells were incubated with the appropriate mAbs for 15 min on ice, and washed with wash buffer between staining reagents. Labeled cells were analyzed on a FACsort flow cytometer using CellQuest software (Becton Dickinson, Mountain View, CA).

Analysis of cytokine production

Purified T cells and T cell subsets were cultured as described above. At 48 h following initiation of culture, cells were harvested from the wells and analyzed for cytokine production by RNase protection assay (RPA). RNA was isolated from cells using TRIzol reagent (Life Technologies; Gaithersburg, MD). Briefly, cells were pelleted and resuspended in 1 ml TRIzol reagent. After 5 min at room temperature, 200 μl $CHCl_3$ was added, samples were mixed well, and then centrifuged at 12,000× g for 15 min at 4°C. The aqueous phase was transferred to a fresh tube and the RNA was precipitated with 500 μl isopropanol. After a 10-min incubation at room temperature, RNA was pelleted and washed with 70% ethanol. RNA pellets were air-dried and resuspended in sterile RNase-free water. RPA was performed on 3 μg of RNA per reaction using a multiprobe RPA (PharMingen) as per manufacturer's instructions.

For analysis of TNF-α production, 50 μl of supernatant was harvested from cultures at 24 h and analyzed by ELISA using a Quantikine M kit (R&D Systems, Minneapolis, MN) as per manufacturer's instructions.

Results

Costimulation through CD81

During the initial characterization of the anti-CD81 mAb, 2F7, it was found that CD81 is expressed on the surface of mature lymphoid T cells and that this expression is increased upon mitogenic T cell stimulation (W. L. Havran and R. Boismenu, unpublished

observations), indicating a possible role for this molecule in T cell activation. This, together with a reported function of CD81 as a costimulatory molecule on human thymocytes (57), raised the possibility that CD81 might also function as a costimulatory molecule on murine mature T cells.

To address this possibility, purified splenic T cells, of which >90% were CD44^{low}, were cultured in microtiter plates that had been coated with anti-CD3 and anti-CD81 mAbs. Coimmobilization of these two Abs induced potent proliferation of the cultured T cells (Fig. 1*A*). This stimulation was comparable to that obtained with coimmobilized anti-CD3 and anti-CD28 mAbs or mitogenic stimulation with PMA and ionomycin (Fig. 1*A*). In contrast, a control mAb (1F3) was unable to induce T cell proliferation when coimmobilized with anti-CD3 mAb, and none of the mAbs alone elicited any stimulation of the T cells (Fig. 1*A*). Comparable costimulation was obtained with splenic T cells from C57BL/6 and BALB/c mice (not shown). Furthermore, costimulation through either CD81 or CD28 induced similar morphology changes in the cultured cells. The cells became enlarged and somewhat irregular shaped, whereas cells ligated with anti-CD3, anti-CD28, or anti-CD81 alone did not show any such changes in morphology. They remained small and round in appearance, as is characteristic of resting T cells (data not shown).

Several of the other molecules described as costimulatory for T cells have been shown to be capable of initiating a proliferative response, but are unable to sustain this response, resulting in apoptosis of the T cells (69). Therefore, we monitored the proliferation and viability of T cells activated via CD81 over a 6-day period. There was remarkable similarity in both proliferation and viability of T cells activated via CD3 and CD28 or CD3 and CD81 throughout the 6 days the T cells were examined (Fig. 1, *B* and *C*). Thus, CD81 appears to be able to provide the costimulatory signal necessary to both initiate and sustain a T cell response.

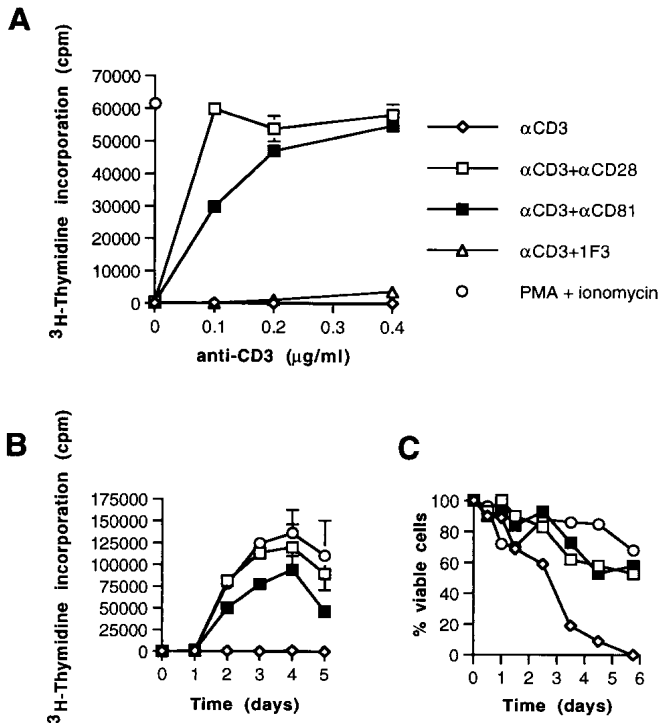

FIGURE 1. T cell response to CD81-mediated costimulation. Purified splenic T cells (1×10^5/well) were cultured in 96-well microtiter plates that had been coated previously with 10 μg/ml of the indicated mAbs, together with increasing concentrations of anti-CD3 mAb. PMA plus ionomycin was used as a mitogen. *A*, [^{3}H]Thymidine incorporation was monitored over the final 14 h of culture and is expressed as the mean cpm ± SD of triplicate cultures. *B*, Proliferation was monitored daily over a 5-day period. For each time point, [^{3}H]thymidine incorporation was measured over the final 14 h of culture. *C*, Cells were cultured for 6 days. At various time points, cells were harvested and viable cells were counted by trypan blue dye exclusion. The data are expressed as the percent of viable cells recovered at each time point.

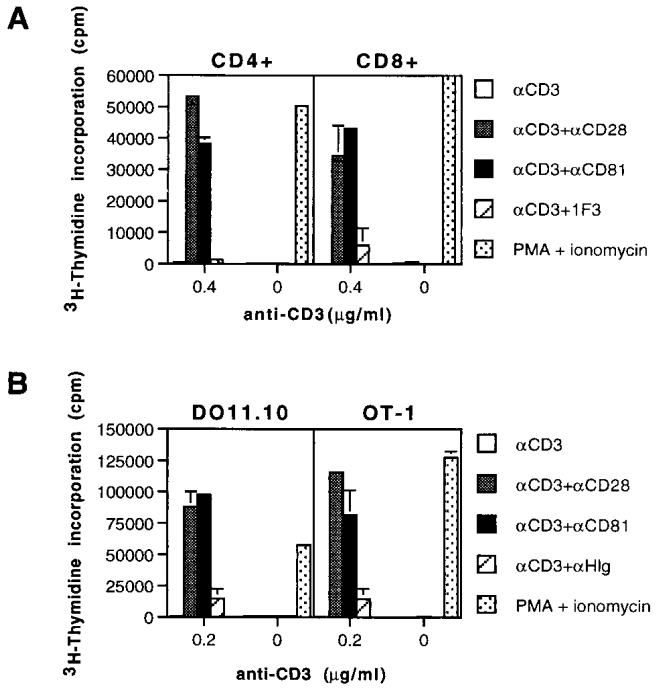

FIGURE 2. T cell subset response to costimulation. Isolated CD4$^+$ and CD8$^+$ splenic T cells (*A*) or RAG$^{0/0}$TCR transgenic CD4$^+$ (DO11.10) and CD8$^+$ (OT-1) T cells (1×10^5/well) (*B*) were cultured in wells containing immobilized Abs as indicated. PMA plus ionomycin was used for mitogenic stimulation. [^{3}H]Thymidine incorporation was monitored over the final 14 h of culture and is expressed as the mean cpm ± SD of triplicate wells.

We then established that costimulation through CD81 was functional in both the CD4$^+$ and CD8$^+$ subsets of T cells. Isolated CD4$^+$ or CD8$^+$ T cells were cultured in microtiter plates that had been coated previously with anti-CD3 and anti-CD81 or anti-CD28. As illustrated in Fig. 2*A*, both CD4$^+$ and CD8$^+$ T cells responded equally well to costimulation whether it was provided by the CD28 or CD81 molecule. Again, a control mAb (1F3) elicited no costimulation in either subset nor did any of the mAbs alone (Fig. 2*A*). This result was confirmed using RAG$^{0/0}$DO11.10 TCR transgenic CD4$^+$ T cells and RAG$^{0/0}$OT-1 TCR transgenic CD8$^+$ T cells (Fig. 2*B*).

Independence of CD81 and CD28

The similarity of the costimulation by anti-CD81 to that seen with anti-CD28 led to the possibility that CD81 may in fact be acting through CD28. This was a distinct possibility in light of the fact that of the many other molecules reported as being costimulatory for T cells, only a few have been shown to act independently of CD28 (26, 39, 41). To address this, we examined the costimulatory ability of anti-CD81 in CD28-deficient (CD28$^{0/0}$) T cells. As shown in Fig. 3*A*, there was no defect in CD81-mediated costimulation in CD28$^{0/0}$ cells when compared with wild-type cells. This was also true of CD81-deficient T cells; there was no defect in CD28-mediated costimulation in the absence of the CD81 molecule (data not shown). Clearly then, CD81 does not require the

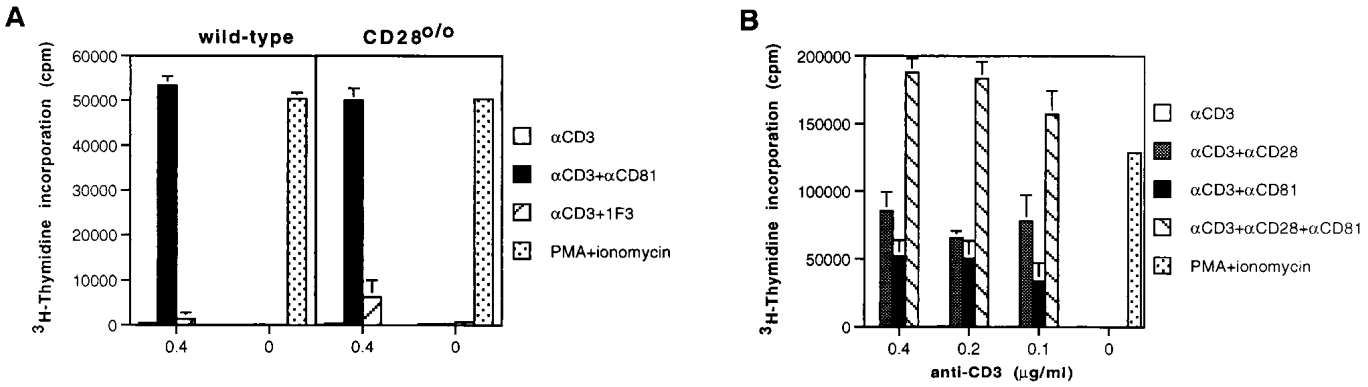

FIGURE 3. Independence of costimulation through CD81 and CD28. *A*, Purified splenic T cells were isolated from wild-type C57BL/6 (wild-type) or CD28-deficient ($CD28^{0/0}$) mice and cultured in wells containing immobilized mAbs as indicated. PMA plus ionomycin was used as a T cell mitogen. [^{3}H]Thymidine incorporation was monitored for the final 14 h of a 72-h culture and is expressed as the mean cpm ± SD of triplicate wells. *B*, Additive effect of costimulation through CD81 and CD28. Purified splenic T cells from C57BL/6 mice were cultured in wells that had been coated previously with increasing concentrations of anti-CD3 together with suboptimal concentrations (2.5 μg/ml) of anti-CD81 and anti-CD28 mAbs either alone or in combination. [^{3}H]Thymidine incorporation was monitored for the final 14 h of a 72-h culture and is expressed as the mean cpm ± SD of triplicate cultures.

presence of CD28 to function, thus costimulation through CD81 functions independently of CD28.

Next we examined the effect of simultaneous costimulation through CD81 and CD28. As illustrated in Fig. 3*B*, using immobilized anti-CD3 with suboptimal amounts (2.5 μg/ml) of anti-CD81 and anti-CD28 together induced proliferation in excess of what was seen with immobilized anti-CD3 in conjunction with either anti-CD28 or anti-CD81 alone. In fact, this simultaneous costimulation induced an additive effect on proliferation, again demonstrating independent stimulation through the CD81 and CD28 molecules.

Although CD28 and CD81 clearly act independently, the possibility remained that these two molecules served the same function and that the intracellular signaling events following ligation of CD28 and CD81 were the same. To address this possibility we made use of two inhibitors, CsA and herbimycin A, which have been reported to differentially effect CD28-mediated costimulation. CsA has been shown to be ineffective as an inhibitor of CD28-mediated costimulation (70), whereas this stimulation is sensitive to the protein tyrosine kinase (PTK) inhibitor, herbimycin A (71–73). Here we found (Fig. 4) that at the concentrations tested, addition of CsA to cultures of freshly isolated splenic T cells had no effect on CD28-mediated costimulation, consistent with previous reports (70). In contrast, increasing concentrations of CsA had a marked effect on costimulation through CD81, inhibiting proliferation by almost half at only 10 ng/ml CsA. Higher concentrations of CsA than shown here were found to be toxic to T cells, and so were not used in these experiments. This differential sensitivity to CsA indicates that not only do CD81 and CD28 function independently, but that their intracellular signaling pathways leading to T cell activation also differ.

Again, in agreement with previous reports (71–73), we found a profound effect of herbimycin A on costimulation through CD28 (Fig. 4). This inhibitory effect also functions for CD81-mediated costimulation as well as mitogenic stimulation with PMA and ionomycin. This indicates a requirement for PTKs in T cell activation via both CD81 and CD28. However, we cannot exclude the possibility that this effect of herbimycin A is in fact on the signals generated through the TCR-CD3 complex, signals that have been well documented to involve PTKs (74).

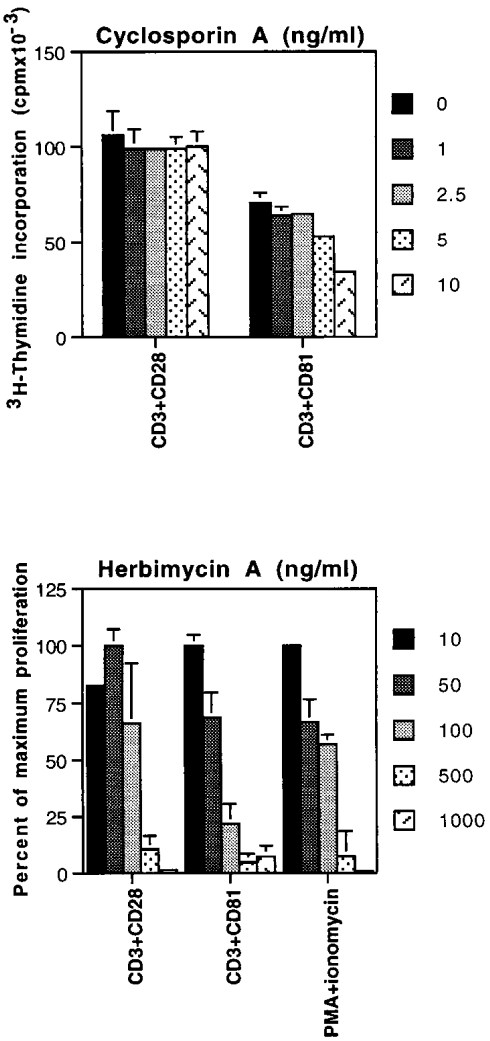

FIGURE 4. Differential effect of inhibitors on CD81- and CD28-mediated costimulation. Purified splenic T cells were cultured in wells containing immobilized mAbs as indicated. CsA or herbimycin A was added at the indicated concentrations. PMA plus ionomycin was also added as indicated. [^{3}H]Thymidine incorporation was monitored for the final 14 h of a 72-h culture.

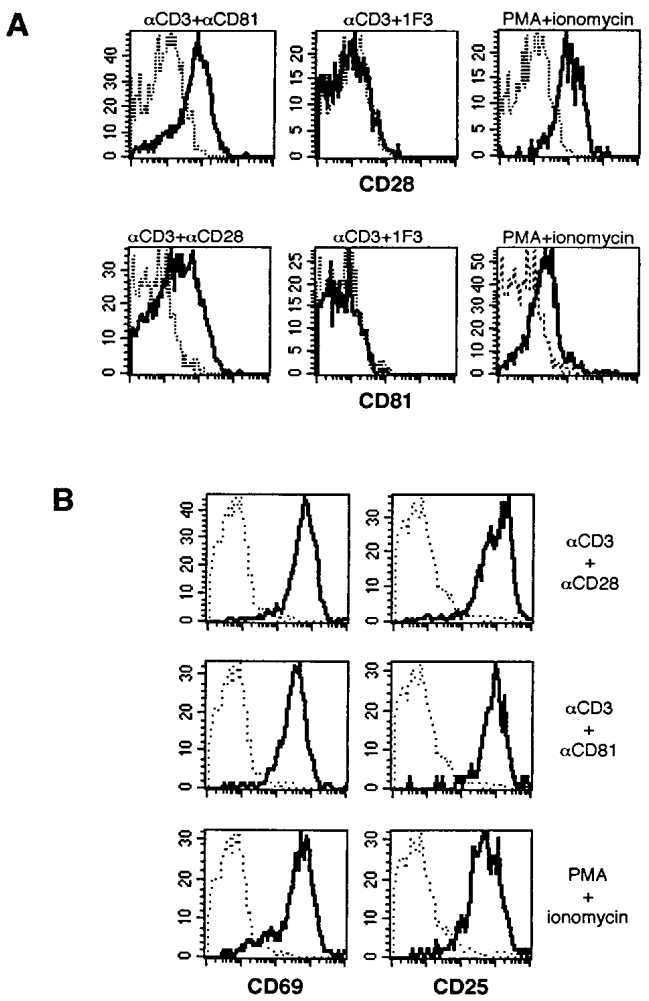

FIGURE 5. Phenotype of activated T cells. Purified splenic T cells were cultured in 96-well microtiter plates that had been coated previously with the indicated mAbs. Cells were harvested at 48 h following initiation of culture and analyzed for the expression of CD28 and CD81 (*A*) and CD69 and CD25 (*B*). Dotted lines represent cells cultured in wells that had not been coated with mAbs.

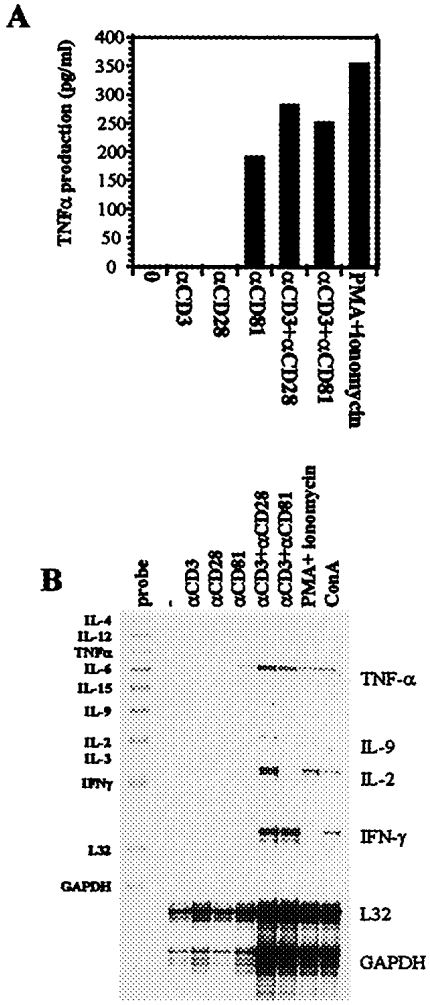

FIGURE 6. Differential cytokine production following CD81- and CD28-mediated costimulation. Purified splenic T cells were cultured in 96-well microtiter plates that had been coated with the indicated mAbs. *A*, Supernatants were harvested at 24 h and assayed by ELISA for the presence of TNF-α. *B*, RNA was isolated from cells harvested at 48 h. Three micrograms total RNA was analyzed for cytokine message by RPA. The major cytokines detected are indicated.

Phenotype of T cells stimulated via CD3 and CD81

Despite the independence of the two signals generated through CD81 and CD28, costimulation of T cells with either anti-CD81 or anti-CD28 or with PMA and ionomycin led to a significant increase in surface expression of both CD81 and CD28, regardless of the stimulus (Fig. 5*A*). A control mAb, which did not induce proliferation (Fig. 1*A*), also did not cause any increase in CD28 or CD81 expression (Fig. 5*A*).

The expression of other molecules associated with activation was also increased following costimulation via CD81 and CD28. As illustrated in Fig. 5*B*, very similar activation profiles were induced regardless of the stimulus. Costimulation via CD81 or CD28 as well as mitogenic stimulation with PMA and ionomycin all led to an increase in expression of the activation markers CD69 and CD25 (Fig. 5*B*) as well as CD44 and a decrease in L-selectin expression (not shown).

Cytokine production following costimulation through CD81

Although the phenotype of cells activated via CD81 or via CD28 was similar, the question remained whether or not these two distinct stimuli translate into equivalent activation of the cells. The differential inhibitory effect of CsA (Fig. 4) suggests that some difference does exist. Furthermore, it has been found in murine (W. L. Havran and R. Boismenu, unpublished observations) and human (75) lymphocytes that ligation of CD81 alone can lead to TNF-α production, whereas this has not been shown for CD28, indicating another difference in the signals generated through these two molecules. To address further this question of differential activation pathways, we examined cytokine production by the activated T cells. In agreement with previous reports, TNF-α was clearly detectable in culture supernatants from cells stimulated with CD81 alone (Fig. 6*A*), and there was some augmentation of this production with CD3 and CD81 costimulation. In contrast, while costimulation through CD3 and CD28 also led to TNF-α production, no TNF-α was produced following stimulation with either CD3 or CD28 alone (Fig. 6*A*).

Further cytokine analysis by multiprobe RNase protection analysis did reveal a second difference in cytokine production (Fig. 6*B*). Although activation of T cells with anti-CD3 together with anti-CD28 led to production of TNF-α, IL-2, IFN-γ, and some IL-9, costimulation through CD81 induced only TNF-α and IFN-γ transcription (Fig. 6*B*). No IL-2 and subsequently no IL-9 was

detectable following costimulation through CD81 (Fig. 6*B*). This result was confirmed by an IL-2-dependent CTLL assay, intracellular cytokine staining, and flow cytometry as well as by RT-PCR (not shown). In addition, IL-2-deficient T cells responded normally to CD81-mediated costimulation and exhibited a diminished response to CD28-mediated costimulation (data not shown). It is thus unclear what cytokine is sustaining the proliferative response to CD81-mediated costimulation. By examining costimulation in IL-10-deficient T cells, we were able to eliminate this cytokine as a possible candidate (data not shown). Nevertheless, taken together, the differential cytokine production following costimulation through CD81 and CD28 suggests distinct functional outcomes of activation via these two molecules.

Discussion

We have demonstrated that the tetraspanin CD81 functions as a potent costimulatory molecule on murine $\alpha\beta$ T cells. Splenic T cells were cultured with coimmobilized anti-CD3 and anti-CD81 mAbs. This led to strong proliferation of both $CD4^+$ and $CD8^+$ T cells, which was comparable in strength and duration to the proliferation elicited by coimmobilized anti-CD3 and anti-CD28 mAbs or mitogenic stimulation with PMA and ionomycin. Consequently we were able to compare and contrast T cell costimulation mediated by CD81 and CD28, and to characterize the mechanism of costimulation through CD81.

The first issue we addressed was the independence of costimulation through CD81 and CD28. Since the identification of CD28 as a costimulatory molecule for T cells, a number of other molecules have been reported to be capable of providing this function (26–35, 38–40). Analysis of mice deficient for CD28 strengthened this idea, as these mice were still capable of mounting efficiently an immune response to several pathogens (24, 25). Nevertheless, of all the alternative molecules described to date, very few have been shown to actually function independently of CD28 (26, 39, 41), although such molecules were presumed to exist given the relatively subtle phenotype of the CD28-deficient mice. We found that costimulation through CD81 did indeed function independently of CD28, and in fact simultaneous costimulation through these two molecules augmented proliferation equivalent to an additive effect of costimulation through CD28 and CD81 independently. Perhaps costimulation via CD81 can account for the ability of CD28-deficient mice to still respond to virus infections and reject allografts. It would be interesting to follow such responses in vivo in animals lacking the CD81 molecule.

Identification of costimulatory molecules functioning independently of CD28 had, to date, been limited to the observation that such molecules can provide a costimulatory signal in cells deficient for the CD28 molecule (26, 39, 41). In this paper we extended our analysis to downstream signals generated through CD81 and CD28. In agreement with what has been reported previously, we found that costimulation through CD28 was insensitive to inhibition by CsA. On the other hand, we observed a strong inhibitory effect of CsA on costimulation through CD81. The transcriptional activation of several cytokine genes (76) and cell surface molecule genes such as CD40L (77) is initiated by NF-AT activation. This activation is blocked by CsA (76, 77). Thus, the inhibitory effect of CsA on costimulation through CD81 indicates a requirement for the Ca^{2+}/calcineurin pathway and NF-AT family members in activation of T cells via CD3 and CD81. As such, it appears that there are different intracellular targets for the signals generated through CD28 and CD81. Some similarities do exist, however. Both costimulatory pathways are sensitive to the PTK inhibitor, herbimycin A. As mentioned earlier, this similarity could be in the signals generated through the TCR rather than through the costimulatory molecule, as PTKs are well documented as being involved in TCR-mediated signaling events (74).

We also observed similarities in the phenotype of the T cells activated via CD3 and CD28 or CD81. CD69, CD25, and CD44 were up-regulated and CD62L was down-regulated regardless of the stimulus used. Interestingly, costimulation through CD81 led to an increase in expression of both CD81 and CD28. Conversely, costimulation through CD28 also resulted in increased expression of both molecules. Together these data demonstrate that costimulation through CD81 results in an activated phenotype, apparently indistinguishable from the phenotype of cells activated by costimulation through CD28.

The phenotypic similarities, yet divergence of intracellular signaling pathways following engagement of CD81 and CD28, led us to question whether the functional outcomes of stimulation through CD81 and CD28 would also diverge. Indeed, cytokine production following ligation of CD81 was quite distinct from that of CD28 engagement. We observed significant TNF-α production following ligation of CD81 alone. This is in agreement with previous observations (W. L. Havran and R. Boismenu, unpublished observations; Ref. 75). No TNF-α was detected following ligation of CD28 alone. This suggests that engagement of the CD81 molecule activates intracellular signals that are unresponsive to CD28 engagement.

IL-2 production is one of the hallmarks of CD28-mediated costimulation (78). Although we also observed strong IL-2 production following costimulation through CD28, we were unable to detect any IL-2 message or protein following costimulation via CD81, again indicating divergence of the signals generated through CD81 and CD28. The lack of IL-2 production following costimulation is not unique to CD81 (41, 43, 69), but is generally associated with an ability to initiate, but not to sustain a proliferative response (69, 79). In the present study, however, costimulation via CD81 led to a vigorous response that was equivalent in extent and duration to a CD28-mediated or mitogenic response. It is unclear what cytokine elicits the proliferative response initiated by costimulation through CD81. From the RPA data, clearly IL-4 and IL-15 are not responsible. We also eliminated IL-10 as a candidate, and thus the molecule or molecules that are responsible for the sustained proliferation and cell survival elicited by CD81-mediated costimulation have yet to be defined. Experiments are currently in progress to characterize further this unique response and its implications in in vivo responses to viruses and other pathogens.

In conclusion, we have demonstrated that CD81 can function as a costimulatory molecule on murine $\alpha\beta$ T cells. This costimulation functions independently of CD28 and, in fact, initiates a different pathway to activation, resulting in a unique functional outcome. Identification of the intracellular pathways activated by CD81 and of a ligand for this molecule on APC would provide insight into its role in in vivo immune responses. It is possible, however, that no ligand exists for CD81, and that the costimulatory effects of CD81 are in fact due to cross-linking of a larger complex. Indeed, CD81 is known to associate with a number of different molecules in human T cells; including CD4, CD8 (55, 56), other TM4SF members, and integrins (44). Thus, the possibility exists that ligation of CD81 leads to signaling through one of its associated molecules rather than directly through CD81 itself. In fact, recent evidence does suggest that TM4SF proteins may act as linkers between extracellular integrin α-chain domains and intracellular signaling molecules such as PI 4-kinase and protein kinase C (61–63).

Whatever the precise mechanisms of CD81-mediated costimulation, clearly CD81 and CD28 act independently and have distinct functional outcomes. However, it does remain unclear whether or

not their activation is initiated by the same stimulus or whether they work together in an in vivo immune response. Interestingly, both CD28- and CD81-deficient mice do have defects in T helper activity (24, 25, 80) suggesting some overlap in their function. Further analysis of immune responses in these deficient animals and in animals lacking both molecules may help clarify their individual roles.

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References

1. Mueller, D. L., M. K. Jenkins, and R. H. Schwartz. 1989. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu. Rev. Immunol. 7:445.*
2. Schwartz, R. H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science 248:1349.*
3. Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, and J. P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature 356:607.*
4. Linsley, P. S., and J. A. Ledbetter. 1993. The role of the CD28 receptor during T cell responses to antigen. *Annu. Rev. Immunol. 11:191.*
5. June, C. H., J. A. Bluestone, L. M. Nadler, and C. B. Thompson. 1994. The B7 and CD28 receptor families. *Immunol. Today 15:321.*
6. Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol. 14:233.*
7. Kersh, E. N., A. S. Shaw, and P. M. Allen. 1998. Fidelity of T cell activation through multistep T cell receptor ζ phosphorylation. *Science 281:572.*
8. June, C. H., J. A. Ledbetter, P. S. Linsley, and C. B. Thompson. 1990. Role of the CD28 receptor in T-cell activation. *Immunol. Today 11:211.*
9. Ward, S. G., J. Westwick, N. D. Hall, and D. M. Sansom. 1993. Ligation of CD28 receptor by B7 induces formation of D-3 phosphoinositides in T lymphocytes independently of T cell receptor/CD3 activation. *Eur. J. Immunol. 23:2572.*
10. August, A., and B. Dupont. 1994. CD28 of T lymphocytes associates with phosphatidylinositol 3-kinase. *Int. Immunol. 6:769.*
11. Prasad, K. V., Y. C. Cai, M. Raab, B. Duckworth, L. Cantley, S. E. Shoelson, and C. E. Rudd. 1994. T-cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr(P)-Met-Xaa-Met motif. *Proc. Natl. Acad. Sci. USA 91:2834.*
12. Truitt, K. E., C. M. Hicks, and J. B. Imboden. 1994. Stimulation of CD28 triggers an association between CD28 and phosphatidylinositol 3-kinase in Jurkat T cells. *J. Exp. Med. 179:1071.*
13. Ueda, Y., B. L. Levine, M. L. Huang, G. J. Freeman, L. M. Nadler, C. H. June, and S. G. Ward. 1995. Both CD28 ligands CD80 (B7-1) and CD86 (B7-2) activate phosphatidylinositol 3-kinase, and wortmannin reveals heterogeneity in the regulation of T cell IL-2 secretion. *Int. Immunol. 7:957.*
14. Boucher, L. M., K. Wiegmann, A. Futterer, K. Pfeffer, T. Machleidt, S. Schutze, T. W. Mak, and M. Kronke. 1995. CD28 signals through acidic sphingomyelinase. *J. Exp. Med. 181:2059.*
15. Chan, G., and A. Ochi. 1995. Sphingomyelin-ceramide turnover in CD28 costimulatory signaling. *Eur. J. Immunol. 25:1999.*
16. Weiss, A., B. Manger, and J. Imboden. 1986. Synergy between the T3/antigen receptor complex and Tp44 in the activation of human T cells. *J. Immunol. 137:819.*
17. Ledbetter, J. A., J. B. Imboden, G. L. Schieven, L. S. Grosmaire, P. S. Rabinovitch, T. Lindsten, C. B. Thompson, and C. H. June. 1990. CD28 ligation in T-cell activation: evidence for two signal transduction pathways. *Blood 75:1531.*
18. Ledbetter, J. A., and P. S. Linsley. 1992. CD28 receptor crosslinking induces tyrosine phosphorylation of PLCγ 1. *Adv. Exp. Med. Biol. 323:23.*
19. Nunes, J. A., Y. Collette, A. Truneh, D. Olive, and D. A. Cantrell. 1994. The role of $p21^{ras}$ in CD28 signal transduction: triggering of CD28 with antibodies, but not the ligand B7-1, activates $p21^{ras}$. *J. Exp. Med. 180:1067.*
20. Siegel, J. N., R. D. Klausner, U. R. Rapp, and L. E. Samelson. 1990. T cell antigen receptor engagement stimulates c-*raf* phosphorylation and induces c-*raf*-associated kinase activity via a protein kinase C-dependent pathway. *J. Biol. Chem. 265:18472.*
21. Su, B., E. Jacinto, M. Hibi, T. Kallunki, M. Karin, and Y. Ben-Neriah. 1994. JNK is involved in signal integration during costimulation of T lymphocytes. *Cell 77:727.*
22. Salojin, K. V., J. Zhang, and T. L. Delovitch. 1999. TCR and CD28 are coupled via ZAP-70 to the activation of the Vav/Rac-1-/PAK-1/p38 MAPK signaling pathway. *J. Immunol. 163:844.*
23. Viola, A., S. Schroeder, Y. Sakakibara, and A. Lanzavecchia. 1999. T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science 283:680.*
24. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science 261:609.*
25. Green, J. M., P. J. Noel, A. I. Sperling, T. L. Walunas, G. S. Gray, J. A. Bluestone, and C. B. Thompson. 1994. Absence of B7-dependent responses in CD28-deficient mice. *Immunity 1:501.*
26. Sperling, A. I., J. M. Green, R. L. Mosley, P. L. Smith, R. J. DiPaolo, J. R. Klein, J. A. Bluestone, and C. B. Thompson. 1995. CD43 is a murine T cell costimulatory receptor that functions independently of CD28. *J. Exp. Med. 182:139.*
27. Liu, Y., B. Jones, A. Aruffo, K. M. Sullivan, P. S. Linsley, and C. A. Janeway, Jr. 1992. Heat-stable antigen is a costimulatory molecule for CD4 T cell growth. *J. Exp. Med. 175:437.*
28. Springer, T. A., M. L. Dustin, T. K. Kishimoto, and S. D. Marlin. 1987. The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. *Annu. Rev. Immunol. 5:223.*
29. Bierer, B. E., A. Peterson, J. C. Gorga, S. H. Herrmann, and S. J. Burakoff. 1988. Synergistic T cell activation via the physiological ligands for CD2 and the T cell receptor. *J. Exp. Med. 168:1145.*
30. Ledbetter, J. A., P. J. Martin, C. E. Spooner, D. Wofsy, T. T. Tsu, P. G. Beatty, and P. Gladstone. 1985. Antibodies to Tp67 and Tp44 augment and sustain proliferative responses of activated T cells. *J. Immunol. 135:2331.*
31. Ceuppens, J. L., and M. L. Baroja. 1986. Monoclonal antibodies to the CD5 antigen can provide the necessary second signal for activation of isolated resting T cells by solid-phase-bound OKT3. *J. Immunol. 137:1816.*
32. Huet, S., H. Groux, B. Caillou, H. Valentin, A. M. Prieur, and A. Bernard. 1989. CD44 contributes to T cell activation. *J. Immunol. 143:798.*
33. Davis, L. S., N. Oppenheimer-Marks, J. L. Bednarczyk, B. W. McIntyre, and P. E. Lipsky. 1990. Fibronectin promotes proliferation of naive and memory T cells by signaling through both the VLA-4 and VLA-5 integrin molecules. *J. Immunol. 145:785.*
34. Nojima, Y., M. J. Humphries, A. P. Mould, A. Komoriya, K. M. Yamada, S. F. Schlossman, and C. Morimoto. 1990. VLA-4 mediates CD3-dependent $CD4^+$ T cell activation via the CS1 alternatively spliced domain of fibronectin. *J. Exp. Med. 172:1185.*
35. Van Seventer, G. A., E. Bonvini, H. Yamada, A. Conti, S. Stringfellow, C. H. June, and S. Shaw. 1992. Costimulation of T cell receptor/CD3-mediated activation of resting human $CD4^+$ T cells by leukocyte function-associated antigen-1 ligand intercellular cell adhesion molecule-1 involves prolonged inositol phospholipid hydrolysis and sustained increase of intracellular Ca^{2+} levels. *J. Immunol. 149:3872.*
36. Saoulli, K., S. Y. Lee, J. L. Cannons, W. C. Yeh, A. Santana, M. D. Goldstein, N. Bangia, M. A. DeBenedette, T. W. Mak, Y. Choi, and T. H. Watts. 1998. CD28-independent, TRAF2-dependent costimulation of resting T cells by 4-1BB ligand. *J. Exp. Med. 187:1849.*
37. Leyton, L., A. F. Quest, and C. Bron. 1999. Thy-1/CD3 coengagement promotes TCR signaling and enhances particularly tyrosine phosphorylation of the raft molecule LAT. *Mol. Immunol. 36:755.*
38. Lebel-Binay, S., C. Lagaudriere, D. Fradelizi, and H. Conjeaud. 1995. CD82, member of the tetra-span-transmembrane protein family, is a costimulatory protein for T cell activation. *J. Immunol. 155:101.*
39. Tai, X. G., Y. Yashiro, R. Abe, K. Toyooka, C. R. Wood, J. Morris, A. Long, S. Ono, M. Kobayashi, T. Hamaoka, et al. 1996. A role for CD9 molecules in T cell activation. *J. Exp. Med. 184:753.*
40. Tai, X. G., K. Toyooka, Y. Yashiro, R. Abe, C. S. Park, T. Hamaoka, M. Kobayashi, S. Neben, and H. Fujiwara. 1997. CD9-mediated costimulation of TCR-triggered naive T cells leads to activation followed by apoptosis. *J. Immunol. 159:3799.*
41. Cocks, B. G., C. C. Chang, J. M. Carballido, H. Yssel, J. E. de Vries, and G. Aversa. 1995. A novel receptor involved in T-cell activation. *Nature 376:260.*
42. Aversa, G., C. C. Chang, J. M. Carballido, B. G. Cocks, and J. E. de Vries. 1997. Engagement of the signaling lymphocytic activation molecule (SLAM) on activated T cells results in IL-2-independent, cyclosporin A-sensitive T cell proliferation and IFN-γ production. *J. Immunol. 158:4036.*
43. Hutloff, A., A. M. Dittrich, K. C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopoulos, and R. A. Kroczek. 1999. ICOS is an inducible T-cell costimulator structurally and functionally related to CD28. *Nature 397:263.*
44. Wright, M. D., and M. G. Tomlinson. 1994. The ins and outs of the transmembrane 4 superfamily. *Immunol. Today 15:588.*
45. Maecker, H. T., S. C. Todd, and S. Levy. 1997. The tetraspanin superfamily: molecular facilitators. *FASEB J. 11:428.*
46. Oren, R., S. Takahashi, C. Doss, R. Levy, and S. Levy. 1990. TAPA-1, the target of an antiproliferative antibody, defines a new family of transmembrane proteins. *Mol. Cell. Biol. 10:4007.*
47. Levy, S., S. C. Todd, and H. T. Maecker. 1998. CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. *Annu. Rev. Immunol. 16:89.*
48. Bradbury, L. E., G. S. Kansas, S. Levy, R. L. Evans, and T. F. Tedder. 1992. The CD19/CD21 signal transducing complex of human B lymphocytes includes the target of antiproliferative antibody-1 and Leu-13 molecules. *J. Immunol. 149: 2841.*
49. Fearon, D. T. 1993. The CD19-CR2-TAPA-1 complex, CD45 and signaling by the antigen receptor of B lymphocytes. *Curr. Opin. Immunol. 5:341.*
50. Fearon, D. T., and R. H. Carter. 1995. The CD19/CR2/TAPA-1 complex of B lymphocytes: linking natural to acquired immunity. *Annu. Rev. Immunol. 13:127.*
51. Carter, R. H., and D. T. Fearon. 1992. CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes. *Science 256:105.*

52. Maecker, H. T., and S. Levy. 1997. Normal lymphocyte development but delayed humoral immune response in CD81-null mice. *J. Exp. Med. 185:1505.*
53. Miyazaki, T., U. Muller, and K. S. Campbell. 1997. Normal development but differentially altered proliferative responses of lymphocytes in mice lacking CD81. *EMBO J. 16:4217.*
54. Tsitsikov, E. N., J. C. Gutierrez-Ramos, and R. S. Geha. 1997. Impaired CD19 expression and signaling, enhanced antibody response to type II T independent antigen and reduction of B-1 cells in CD81-deficient mice. *Proc. Natl. Acad. Sci. USA 94:10844.*
55. Imai, T., and O. Yoshie. 1993. C33 antigen and M38 antigen recognized by monoclonal antibodies inhibitory to syncytium formation by human T cell leukemia virus type 1 are both members of the transmembrane 4 superfamily and associate with each other and with CD4 or CD8 in T cells. *J. Immunol. 151:6470.*
56. Imai, T., M. Kakizaki, M. Nishimura, and O. Yoshie. 1995. Molecular analyses of the association of CD4 with two members of the transmembrane 4 superfamily, CD81 and CD82. *J. Immunol. 155:1229.*
57. Todd, S. C., S. G. Lipps, L. Crisa, D. R. Salomon, and C. D. Tsoukas. 1996. CD81 expressed on human thymocytes mediates integrin activation and interleukin 2-dependent proliferation. *J. Exp. Med. 184:2055.*
58. Levy, S., V. Q. Nguyen, M. L. Andria, and S. Takahashi. 1991. Structure and membrane topology of TAPA-1. *J. Biol. Chem. 266:14597.*
59. Berditchevski, F., S. Chang, J. Bodorova, and M. E. Hemler. 1997. Generation of monoclonal antibodies to integrin-associated proteins: evidence that $\alpha_3\beta_1$ complexes with EMMPRIN/basigin/OX47/M6. *J. Biol. Chem. 272:29174.*
60. Tachibana, I., J. Bodorova, F. Berditchevski, M. M. Zutter, and M. E. Hemler. 1997. NAG-2, a novel transmembrane-4 superfamily (TM4SF) protein that complexes with integrins and other TM4SF proteins. *J. Biol. Chem. 272:29181.*
61. Berditchevski, F., K. F. Tolias, K. Wong, C. L. Carpenter, and M. E. Hemler. 1997. A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. *J. Biol. Chem. 272:2595.*
62. Hemler, M. E. 1998. Integrin associated proteins. *Curr. Opin. Cell Biol. 10:578.*
63. Yauch, R. L., F. Berditchevski, M. B. Harler, J. Reichner, and M. E. Hemler. 1998. Highly stoichiometric, stable, and specific association of integrin $\alpha_3\beta_1$ with CD151 provides a major link to phosphatidylinositol 4-kinase, and may regulate cell migration. *Mol. Biol. Cell 9:2751.*
64. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+TCR^{lo}$ thymocytes in vivo. *Science 250: 1720.*
65. Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell 76:17.*
66. Gross, J. A., E. Callas, and J. P. Allison. 1992. Identification and distribution of the costimulatory receptor CD28 in the mouse. *J. Immunol. 149:380.*
67. Boismenu, R., M. Rhein, W. H. Fischer, and W. L. Havran. 1996. A role for CD81 in early T cell development. *Science 271:198.*
68. Wysocki, L. J., and V. L. Sato. 1978. "Panning" for lymphocytes: a method for cell selection. *Proc. Natl. Acad. Sci. USA 75:2844.*
69. Yashiro, Y., X. G. Tai, K. Toyo-oka, C. S. Park, R. Abe, T. Hamaoka, M. Kobayashi, S. Neben, and H. Fujiwara. 1998. A fundamental difference in the capacity to induce proliferation of naive T cells between CD28 and other costimulatory molecules. *Eur. J. Immunol. 28:926.*
70. June, C. H., J. A. Ledbetter, M. M. Gillespie, T. Lindsten, and C. B. Thompson. 1987. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Mol. Cell. Biol. 7:4472.*
71. Lu, Y., A. Granelli-Piperno, J. M. Bjorndahl, C. A. Phillips, and J. M. Trevillyan. 1992. CD28-induced T cell activation: evidence for a protein-tyrosine kinase signal transduction pathway. *J. Immunol. 149:24.*
72. Vandenberghe, P., G. J. Freeman, L. M. Nadler, M. C. Fletcher, M. Kamoun, L. A. Turka, J. A. Ledbetter, C. B. Thompson, and C. H. June. 1992. Antibody and B7/BB1-mediated ligation of the CD28 receptor induces tyrosine phosphorylation in human T cells. *J. Exp. Med. 175:951.*
73. Los, M., H. Schenk, K. Hexel, P. A. Baeuerle, W. Droge, and K. Schulze-Osthoff. 1995. IL-2 gene expression and NF-κB activation through CD28 requires reactive oxygen production by 5-lipoxygenase. *EMBO J 14:3731.*
74. June, C. H., M. C. Fletcher, J. A. Ledbetter, G. L. Schieven, J. N. Siegel, A. F. Phillips, and L. E. Samelson. 1990. Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. *Proc. Natl. Acad. Sci. USA 87:7722.*
75. Altomonte, M., R. Montagner, C. Pucillo, and M. Maio. 1996. Triggering of target of an antiproliferative antibody-1 (TAPA-1/CD81) up-regulates the release of tumour necrosis factor-α by the EBV-B lymphoblastoid cell line JY. *Scand. J. Immunol. 43:367.*
76. Crabtree, G. R., and N. A. Clipstone. 1994. Signal transmission between the plasma membrane and nucleus of T lymphocytes. *Annu. Rev. Biochem. 63:1045.*
77. Fuleihan, R., N. Ramesh, A. Horner, D. Ahern, P. J. Belshaw, D. G. Alberg, I. Stamenkovic, W. Harmon, and R. S. Geha. 1994. Cyclosporin A inhibits CD40 ligand expression in T lymphocytes. *J. Clin. Invest. 93:1315.*
78. Fraser, J. D., B. A. Irving, G. R. Crabtree, and A. Weiss. 1991. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science 251:313.*
79. Lucas, P. J., I. Negishi, K. Nakayama, L. E. Fields, and D. Y. Loh. 1995. Naive CD28-deficient T cells can initiate but not sustain an in vitro antigen-specific immune response. *J. Immunol. 154:5757.*
80. Maecker, H. T., M. S. Do, and S. Levy. 1998. CD81 on B cells promotes interleukin 4 secretion and antibody production during T helper type 2 immune responses. *Proc. Natl. Acad. Sci. USA 95:2458.*